A frightening view on schizophrenia. Combining fear conditioning and ketamine administration to investigate emotional blunting in an animal model of schizophrenia

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Chapter 2

Fear conditioning and shock intensity: The choice between minimising the stress induced and reducing the number of animals used

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Abstract

Many fear conditioning studies use electric shock as the aversive stimulus. The intensity of shocks varies throughout the literature. In this study, shock intensities ranging from 0mA to 1.5mA were used, and the effects on the rats assessed by both behavioural and biochemical stress parameters.

Results indicated a significant difference with respect to defecation and freezing behaviour between controls and those animals that received a shock. Significant differences in corticosterone levels were also noted between controls and those groups that received a shock.

No significant differences were found between the shock groups with regards to the stress parameters measured in our fear-conditioning paradigm, indicating that the two shock groups were similarly stressed. Increased significance levels were noted for freezing behaviour as well as a lower standard error of means was found in the highest shock intensity group.

We therefore recommend using the higher shock intensity (1.5mA) as the rats in the higher shock intensity group were more homogenously fear-conditioned and therefore the results should be more reproducible and robust than in the lower shock intensity group. This would allow for fewer rats to be used in order to gain an accurate impression of the conditioning paradigm employed.

1 Introduction

Classic fear conditioning is used throughout the literature to study fear and reward circuits in the brain (Maren, 2001; Gerrits et al., 2003; Li et al, 2004). It typically involves pairing a neutral conditioned stimulus with an aversive unconditioned stimulus. After a few trials, the conditioned stimulus then elicits the same response as the aversive stimulus (Walker and Davis, 2002), namely the conditioned response. By measuring the freezing behaviour of the animals in response to the conditioned stimulus, one can then determine whether fear conditioning was acquired or not. However, many discrepancies occur in the various studies concerned. This extends from the type and intensity of the aversive stimuli employed to the number of times an animal is conditioned (1 day to 21 days) (Kaehler et al., 2000; Pezze et al., 2002; Gerrits et al., 2003; Kikusui et al., 2003).

Fear conditioning is also considered to be stress inducing (Sotty et al., 1996; Suzuki et al., 2002). Accordingly, the stress the animal experiences, may be a good indicator of fear response. This is especially true when it is measured after conditioning and in the absence of physical stressors. Essentially the emotional stress reaction in response to the conditioning can then be obtained.

We were interested in measuring the stress that the animal experiences, not only to determine whether fear conditioning was acquired, but also to determine quantitatively what the animal endures. The Hypothalamic-Pituitary-Adrenal (HPA) axis is the pathway through which activation by a stressor ultimately leads to the release of cortisol (in humans) or corticosterone (in animals) (Nash and Maickel, 1988; Udelsman and Chrousos, 1988). Therefore, in order to determine the stress effect of the different shock intensities induced in our paradigm, plasma corticosterone levels were measured.

The behavioural parameters, freezing and defecation, are also commonly used as indicators of stress (Goldstein et al., 1996; Suzuki et al., 2002) and were taken as such in our study. They are also indicative of fear or anxiety (Babar et al., 2001; Goldstein et al., 1996). Freezing in particular is the key behaviour to determine

whether fear conditioning has been obtained or not (Inoue et al., 1993; Holahan et al., 2002; Levita et al., 2002).

The aim of this study was to investigate the effects of classic pavlovian fear conditioning on various stress parameters in order to quantify the stress experienced by the rat as well as the relation between the variance of the behaviour shown to the shock intensity delivered. We therefore wanted to determine which shock intensity causes the least amount of stress to the animal undergoing an acute fear conditioning paradigm, as well as optimising the results obtained.

2 Materials and methods

2.1 Animals

Male Sprague-Dawley rats (outbred strain, Harlan, The Netherlands) (n=15) weighing between 225-250g were obtained from the central animal facility (Groningen, The Netherlands). The animals were tested for viruses, bacteria, mycoplasma, fungi, parasites and pathological lesions and were found to be healthy and specified pathogen free. After arrival from the animal breeding facility, they were housed individually and conventionally in cages (38x22x18cm, lxbxh) with enriched environments (wooden stick, tissue paper) and allowed to acclimatise for two to three days. They were then handled daily for five days for five minutes per day in order to eliminate handling stress as a confounding variable. The treatment schedule and protocol were approved by the local animal experimental commission (DEC: Dierenexperimentele commissie, Groningen, The Netherlands), under the law for the care of experimental laboratory animals (Experiment number: DEC 2823).

2.2 Husbandry during experiment

Animals were housed individually in perspex cages lined with sawdust bedding (LTE E-001, ABEDD) in a temperature (± 23℃) and humidit y controlled (40 to 60 %) conventional environment. They were kept on a 12-hour light/dark cycle, with the light cycle beginning at 7am, ending at the beginning of the dark cycle at 7pm. Ventilation in combination with air conditioning was provided by means of an outlet filtration system. Methods to refine experimental techniques were applied in accordance with

the three R's (replacement, reduction, refinement). In order to determine the effects on the whole organism, the animals could not be replaced with other techniques.

2.3 Feeding

Food (pellets) obtained from Hope farms (RMH-B, Woerden, The Netherlands) was given to the animals and consisted of a normal grain diet enriched with dietary vitamins, the composition of which is given in Table 1. Water was obtained from normal taps suitable for human consumption. Both food and water were available *ad lib.*

Table 1 The chemical analysis and vitamin content of rat pellet food

RMH-B rodent chunks 2181	
Analysis (%)	
24.0	Raw protein
5.5	Raw fat
4.0	Cellulose
5.6	Raw ash
0.65	Calcium
0.49	Total phosphor
10.0	Fluid
0.4	Sodium
0.88	Potassium
0.12	Magnesium
Vitamins	
20600 IE/kg	A
1900 IE/kg	D_3
95 IE/kg	C
60 IE/kg	E
11 mg/kg	Copper

2.4 Experimental Procedure

A total of 15 rats were used in this experiment. The animals were divided into 3 groups: controls (n=5), fear conditioned with 1,0mA (n=5) and fear conditioned with 1,5mA (n=5) as several behavioural studies indicate that five animals are adequate in order to obtain significant results (Wilensky et al, 2000; Majak and Pitkanen, 2003). The rats were taken out of their home cage and placed individually in the shock box. This was a specially constructed wooden container (25x25x25cm) with a floor made of a metal grid. A central computer controlled the current and noise emission making use of a programme that was specially developed for this study (N594 ver. 2.00,

Rijksuniversteit Groningen, The Netherlands, 2002). The DC current was scrambled and randomised across the individual bars. Rats (n=10) were then subjected to fear conditioning on two consecutive days by means of pairing a noise (60dB, pure tone) with a shock (1.0 or 1.5mA). One shock session consisted of a 1-minute period (Fig. 1) that was repeated consecutively ten times per day. All sessions took place in the morning (± 10am) and lasted 10 minutes in total.

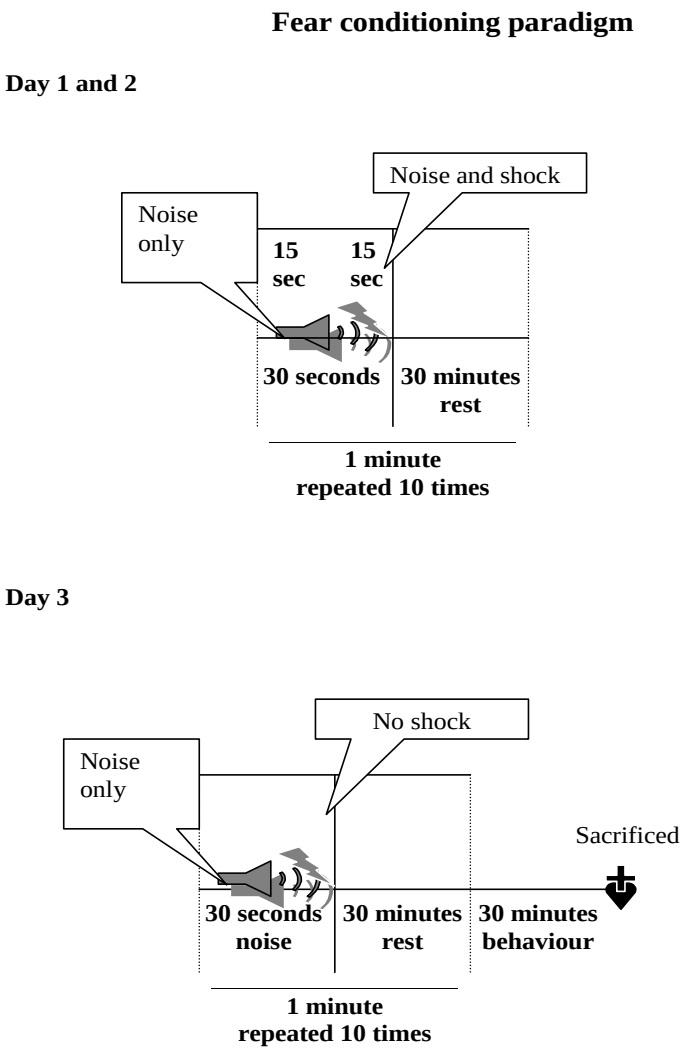

Figure 1: *Fear conditioning paradigm. This diagram is representative of the 1-minute shock session that is repeated 10 times per day on day 1 and day 2. On day 3, the same process is followed, but without administering the shocks, where after the behaviour is noted and the rats are sacrificed via decapitation.*

On the third day, the same procedure was followed, but without administering the shocks. The behaviour was then noted for 30 minutes after the last noise session. Control animals received the same treatment without receiving the shocks (n=5).

2.5 Behavioural measurements

Behaviours were recorded for each rat by means of a video camera and recorder (Philips Explorer Camcorder) directly after the last noise session for 30 minutes. They were then subsequently analysed with the aid of the computer programme, The Observer (Noldus version 3.0, The Netherlands). Freezing as well as defecation (total number of faecal boli) was noted. The rats were then sacrificed by means of decapitation (after a brief exposure to 70% CO_2 (in oxygen or air) for a quick loss of consciousness without hypoxia) and trunk blood was taken for the measurement of corticosterone levels.

2.6 Corticosterone levels

Plasma corticosterone levels were measured by HPLC with UV detection (Trentani et al., 2002). Dexamethasone was used as an internal standard during corticosterone quantification. Plasma was extracted with 3ml of diethylether, vortexed for 5 minutes and then centrifuged for five minutes at 3000g at 4℃. The organic phase was carefully removed and then evaporated to dryness in a 50 ℃ waterbath. The detection limit of corticosterone was 10nM/ 0,35µg/dl.

2.7 Statistics

After establishing homogeneity of variances and a normal distribution, the main effect of fear conditioning as a group factor was assessed between the three groups (0, 1.0, 1.5mA) with a univariate analysis of variance, one-way ANOVA (SPSS package, version 10). When a significance of $p < 0.05$ was found, Tukey's posthoc was applied to the data. Here too significance was taken at $p < 0.05$. Defecation data did not show a normal distribution and therefore a Kruskal-Wallis non-parametric test was performed to determine main effects of fear conditioning with a Mann-Whitney test as post hoc. In both cases significance was determined at $p<0.05$.

3 Results

3.1 Behavioural Measurements

The behavioural data indicated that those animals that received a shock exhibited more stress-related behaviours, including an increase in the total amount of time spent freezing and in defecation. Statistically significant main effects of fear

conditioning were noted between the groups with respect to defecation (χ^2 =9,015; Df = 2; p<0.011). Mann-Whitney post-hoc tests revealed significant differences between controls (n=5) and those animals that received a shock intensity of 1.0mA (n=5; p=0.008) (fig. 2) or 1.5mA (n=4; p=0.016). However no differences were noted between the two shock groups (p=0.73).

Differences between the groups were also noted with respect to total duration of freezing behaviour displayed ($F_{2,12}$=7.09, p=0.009) with controls being significantly lower than the 1.0mA (p=0.032) and 1.5mA (p=0.011) shock intensity groups (Fig. 2). No differences were found between the two shock intensity groups in this parameter (p=0.832).

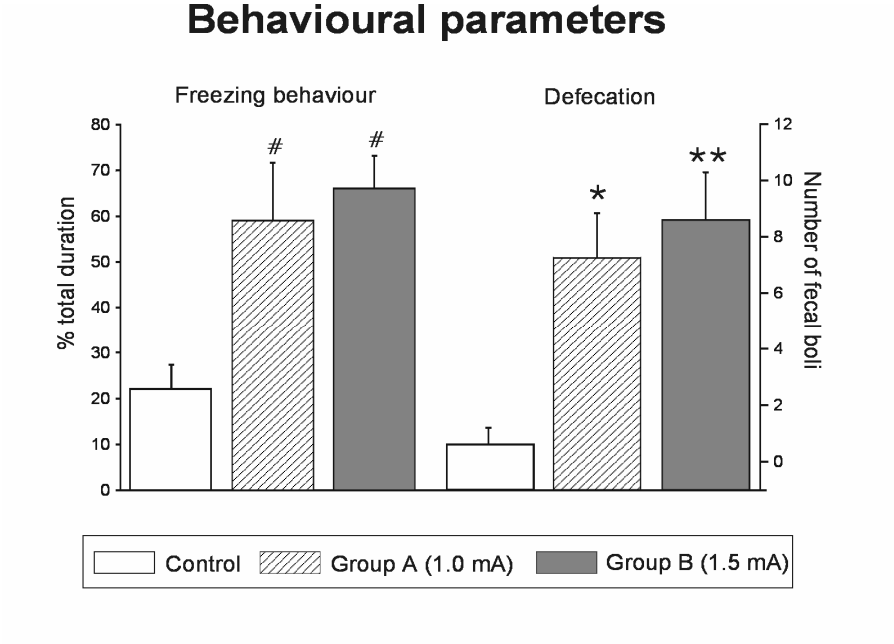

***Figure 2**: Behavioural parameters. Behavioural parameters are depicted here as mean ± SEM for total freezing duration and the median with interquartile boxes and full range whiskers for defecation. Group B (1.5mA, n = 5) and group A (1.0mA, n = 5) displayed increased freezing behaviour as compared to controls (0mA, n = 5) ($^{\#}p<0.05$). No differences were noted between shock groups (p=0.83). With regards to defecation, both groups again (A: n = 5, $^{\#\#}p<0.01$, B: n = 4, $^{\#}p<0.05$) showed an increased amount of faecal boli excreted as compared to controls (n = 5). The shock groups did not significantly differ from each other (p=0.73). The asterisk* represents an outlier.*

3.2 Corticosterone levels

Statistical differences ($F_{2,11}$=14.46, p=0.001) were noted in corticosterone levels between controls and those groups that received a shock (A: n=5, p=0.002; B: n=4, p=0.002), but again no differences were found between the two different shock intensity groups (p=0.950, Fig. 3). A lower standard error of the mean was however noted in the 1.5mA group. One sample in the 1.5mA group was disregarded due to analytical failure.

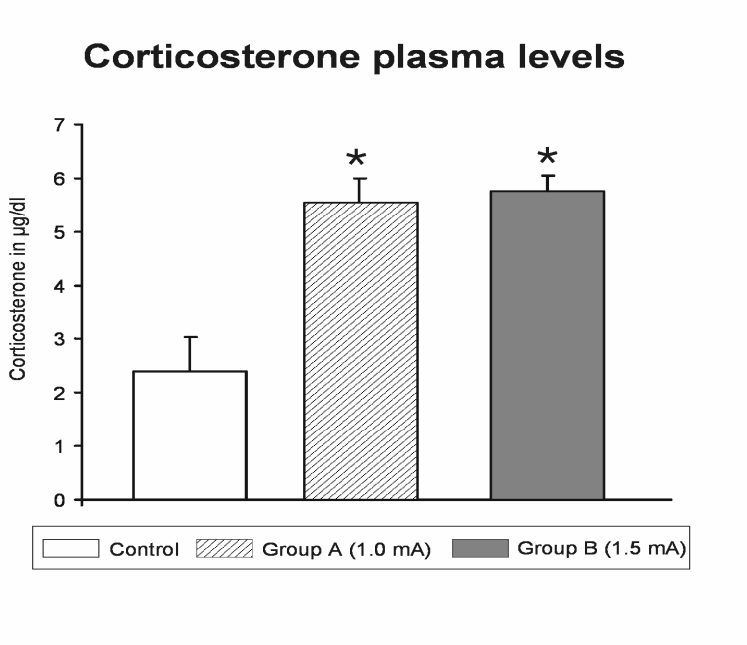

***Figure 3**: Corticosterone plasma levels. Corticosterone levels are depicted here as the mean ± SEM. The two shock groups (A: n = 5, B: n = 4) have significantly higher corticosterone levels (*p=0.002) when compared to controls (n = 5). Again, no significant differences between the two shock groups were found (p=0.95).*

4 Discussion

The aim of this study was to investigate the effects of two different shock intensities in the context of classical pavlovian fear conditioning on various stress parameters in order to determine its effects quantitatively on the animal. We approached this aim by aversively conditioning rats to a sound for 2 days, with no shock being given on the third day in order to evaluate only the psychological effects of the conditioning on freezing, defecation behaviour and corticosterone levels.

The main finding of our study was that those rats that were subjected to fear conditioning with our acute shock paradigm, were indeed stressed and thereby fear conditioned (Goldstein et al., 1996; Suzuki et al., 2002). Our behavioural results show increased freezing and defecation behaviour (Fig. 2). However, as the rats did not receive any shocks on the day of measurement, we can deduce that the behaviour displayed was emotional and in anticipation of a stressful event associated with the tone emitted and therefore not based on the painful stimulus itself, thus indicating that they were indeed fear conditioned.

In both shock intensity groups, a significant increase in corticosterone levels was noted (Fig. 3), indicating that these two groups were more stressed than the control

group (Paris et al., 1987; Pitman et al., 1995; Codero et al., 2002). The fact that there was no difference between the two shock intensity groups, suggests that the rats in these two groups were equally stressed, or that the maximum stress threshold (ceiling) had been reached, and therefore an increase of 0.5mA in shock intensity did not have a significant effect on corticosterone levels between the two groups. Either shock intensity, based on this result, could therefore be employed without imposing severe consequences on the amount of corticosterone released or stress endured.

Looking at defecation, another stress marker (Goldstein et al., 1996, Suzuki et al., 2002), both shock groups were again significantly increased as compared to controls (Fig.2) with the two shock groups not differing from each other. We therefore conclude that the rats subjected to fear conditioning with electric shocks of varying intensity (1.0mA or 1.5mA) experienced the same amount of stress.

Freezing behaviour is considered to be the most reliable output measure of fear conditioning (Inoue et al., 1993; Holahan et al., 2002; Levita et al., 2002). In our paradigm, no differences were noted between shock groups with respect to freezing behaviour, indicating that our rats were similarly stressed and conditioned. The standard error of the mean however, appears to be smaller in the higher shock intensity group compared to the lower shock intensity group (fig. 2). The higher shock intensity group also displayed an increased significance level compared to controls than the lower shock intensity group ($p=0.032$ in the 1.0mA vs. $p=0.011$ in the 1.5mA group).

Although this study does limit itself to changing only one parameter, shock intensity, and several other parameters would also have an effect on the robustness of the fear conditioning, our results suggest that the rats in the higher shock intensity group were more homogenously fear-conditioned and therefore the results should be more reproducible and robust than in the lower shock intensity group. This would allow for fewer rats to be used in order to gain an accurate impression of the conditioning paradigm employed.

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